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13. ABSTRACT *(Maximum 200 Words)*
Extracellular matrix proteins (ECM) play a key role in tissue remodeling and tumor progression. We have shown that matrix metalloproteinase (MMP-2) cleaves Ln-5 and promotes cell migration. We report here that the expression of MMP-2 and the cleavage of Ln-5 correlate with the tissue remodeling of the mammary gland in vivo and in vitro as well. Sex steroids stimulate the development of the gland, with the occurrence of new buds and branches. In this situation Ln-5 was detected in the cleaved form, and MMP-2 concentrated along the mammary epithelial cells penetrating in the fat pad. A remodeling of the mammary gland tissue with a concomitant cleavage of Ln-5 was also observed in cultured mammary gland tissue These results suggest that a cleavage of Ln-5 by MMP-2 might be regulated by sex steroids and that this could contribute to tissue remodeling and cancer metastasis. An alternative possibility is that MT1-MMP could cleave Ln-5, in those cells that do not express MMP-2. We demonstrated that MT1-MMP is also able to promote cell migration via Ln-5 cleavage. Several evidences suggest that this mechanism might be largely diffuse in cancer tissues and the cleavage of Ln-5 could be relevant for tumor progression and metastasis.

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Table of Contents

INTRODUCTION.

Occurrence of metastasis in course of breast cancer is a common event that affect long-term prognosis. No therapy is currently available to prevent or to block breast cancer spread, mainly because the mechanisms that control tumor invasion are still unknown. Cancer invasion and metastasis are a complicate process that involves several steps(1). Cancer cells need to cross the basal membrane in order to penetrate the surrounding tissue and to spread into blood vessels. During this process cells interact with extracellular matrix (ECM) components. It is general idea that proteases such as Matrix Metalloproteases (MMPs), play a key role in remodeling ECM proteins facilitating cancer cell progression (2;3).
An ECM component, Laminin-5 (Ln-5), a Laminin isoform expressed at basal membrane level has a crucial role in tissue omeostasis (4). Ln-5 promotes static adhesion and partecipate in hemidesmosome formation (5). Our preliminary results, indicate that MMP-2 cleaves Ln-5 and convert this substrate from an adhesive one into a migratory (6) (7). In our opinion this finding could be relevant because MMP-2 cleaves Ln-5 and breast cells acquire motility.
Our goal is to explore the possibility that the cleavage of Ln-5 could be relevant in breast cancer cell motility and invasion.

BODY OF PURPOSE.

We have previously described a mechanism in vitro by which Ln-5 is cleaved by MMP-2 and promotes breast cell migration. In the course of my research training I have achieved two main goals have been:

1) To investigate the role of the mechanism previously described in vivo;
2) To investigate additional mechanism for promoting cell motility.

To investigate the role of the MMP-2 cleaved Ln-5 we have used an in vivo model. In particular we have used as model the tissue remodeling that occur in the mammary gland. It is well known that the gland undergoes during life to dramatic remodeling from the puberty to the involution. During the development of the mammary gland, branch morphogenesis occurs (8). Mammary gland cells cross the basement membrane, and penetrate the fat pad creating new ducts. The mechanism used by the mammary cells is the same that is used by cancer cells. It is well known that mammary gland development is under sex steroids control. In this research we focused on the mechanism by which mammary gland penetrate the fat pad. We observed that Ln-5 was found intact during puberty and lactation state, thus in those phase when the gland does not undergo to tissue remodeling. On the contrary with the beginning of ovarian cycle and the appearing of sex hormones Ln-5 was found in a cleaved form, as well as during pregnancy and during involution. The development of the mammary gland initiates with the beginning of the ovarian cycle, it increases during pregnancy when the gland is growing for the lactation. During this period the development of the gland is stopped, and start again with the involution so new branches appear to prepare the gland for a new lactation. In sex immature rats, when the gland is not yet developed, we stimulate the growth of the gland with exogenous sex hormones. At the end of the treatment we analyzed the development of the gland by a whole mount. After this treatment, new branches and penetration in the fat pad was evident. Interestingly, the cleaved form of Ln-5 was detected in the mammary gland of rats treated with sex steroids but not in the controls. MMP-2 was also investifated by immunohistochemistry. A positive staining was observed around new branches and new buds in the mammary gland of the sex steroids treated animals, while it was completely absent in the untreated rats. Furthermore, we observed that mammary gland tissues cultured in presence of MMP-2 presented a detachment of epithelial cells and a remodeling of the gland. In these preparations Ln-5 was detected in the cleaved form. We abolished the MMP-2 effects by using a matrix metallopreases inhibitor such as BB-94. In these samples, no cells were detached and Ln-5 was detected in the uncleaved form as in the controls. Finally, we observed that human Ln-5 was cleaved by MMP-2 and also that the cleaved form promoted cell scattering (9).

Based on those results we proposed a mechanism by which sex steroids control the growth and the development of the mammary gland via an increased secretion of MMP-2, that could cleave Ln-5 and stimulate cell invasion. Thus, this mechanism could be used also by cancer cells in order to invade and metastasize surrounding tissue. In particular, the regulation of sex steroids could have a crucial role since anti-estrogen drugs are currently used in breast cancer. Several in vitro studies reported that estrogens block cell migration and invasion through a reconstituted basement membrane.

Tissue remodeling occurs in several medical situations, both physiological and pathological. Growth of uterus during pregnancy is just an example of tissue remodeling, but also wound healing and mammary gland development represent typical situation of tissue remodeling. Similarly, cancer development and tumor spread are also situation where remodeling occurs. In this contest the cell-extracellular matrix protein contact has a crucial role in controlling and regulating cell functions. Matrix metalloproteinases play a central role since they can degrade the ECM components and facilitate the remodeling of tissues by simply removing physical barrier. An other possibility is that MMPs can cleave ECM components and change their biological functions. This is the case of Ln-5, and this is the topic of the described findings.

We also observed that MMP-2 was concentrated along the new buds and branches, however no positive staining was evident in the mammary epithelial cells, while a positive staining was observed in the stroma. This observation was very interesting, since it not yet clear whether mammary epithelial cells are able to produce and/or activate MMP-2. In other system MMP-2 is activated by stromal cells and released so that the cancer cells can capture the activated enzyme and use it to migrate and invade. We investigated this hypothesis in different epithelial cells. Interestingly we found that cells that do not secrete detectable levels of MMP-2 were still able to migrate on Ln-5. This migratory capability was inhibited by BB-94 and by TIMP-2 both able to bock MMPs activity. We furthermore investigated the plasma membrane type-1 metalloproteinase (MT1-MMP) and we found that it was expressed in the plasma membrane preparations of those cell types. MT1-MMP has a key role in MMP-2 activation since with TIMP-2 it forms a complex that binds MMP-2 at the cellular surface activating MMP-2. However, MT1-MMP can also directly degrade ECM components such as Ln-5. This was proved in an other study by several evidences. Soluble MT1-MMP was able to cleave Ln-5 with a similar pattern previously described for MMP-2; cancer cell migration was inhibited by MT1-MMP antisense oligonucleotides; cell scattering was also blocked by MT1-MMP antisense oligonucleotides. All these results suggest a model whereby MT1-MMP expression can directly induce cell migration over Ln-5 because the γ2 cleavage or MMP-2 activation, while MMP-2 may play an additional role amplifying the MT1-MMP activity. Codistribution of MT1-MMP with Ln-5 in colon and breast cancer tissue also suggest a role for this mechanism in invasion. Thus, Ln-5 cleavage by Mti-MMP/MMP-2 complex may represents a common and largely diffuse mechanism that triggers cancer cell migration and invasion (7).

During this period I have been trained in biological, and biochemical fields, but also I learned new techniques to deal with tissue and with in vivo model. I had experiences in other laboratories for short visit that contributed to my scientific growth. For all these reasons I believe that this award has been a great opportunity for my research and also I believe that the results I obtained during this training period are very important in the migration and metastasis field.

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Expression of Matrix Metalloprotease-2-Cleaved Laminin-5 in Breast Remodeling Stimulated by Sex Steroids

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The extracellular matrix plays an important role in breast remodeling. We have shown that matrix metalloprotease-2 (MMP2) cleaves laminin-5 (Ln-5), a basement membrane component, generating a fragment called γ2x. Human breast epithelial cells, while constitutively immobile on intact Ln-5, acquire a motile phenotype on MMP2-cleaved Ln-5. We hypothesize that this mechanism may underlie cell mobilization across the basement membrane during branching morphogenesis in breast development regulated by sex steroids. We report that the expression of MMP2 and cleavage of Ln-5 correlate well with tissue remodeling and epithelial rearrangement of the breast both *in vivo* and *in vitro*. Thus, the Ln-5 γ2x fragment was detected by immunoblotting in sexually mature, pregnant, and postweaning, but not in prepubertal or lactating mammary glands. Furthermore, cleaved Ln-5, as well as MMP2, became detectable in remodeling glands from sexually immature rats treated with sex steroids. In rat mammary gland explants, epithelial reorganization and luminal cell morphological changes were induced by the addition of exogenous MMP2, in parallel to the appearance of cleaved Ln-5. Similar effects were observed in epithelial monolayers plated on human Ln-5 and exposed to MMP2. These results suggest that cleavage of Ln-5 by MMP2 might be regulated by sex steroids and that it may contribute to breast remodeling under physiological and possibly pathological conditions. *(Am J Pathol 1999, 154:1193–1201)*

Tissue organization and specialization is a challenging issue in medical science. In many physiological and pathological conditions such as tissue repair and tumor invasion, tissue architecture and cell-stroma boundaries are rearranged, resulting in the reformation of pre-existing structures or in the generation of new structures. This process, commonly known as tissue remodeling, requires the interaction between epithelium and mesenchyma mediated, at least in part, by extracellular matrix (ECM) proteins. ECM components, including laminin-1 and laminin-5 (Ln-5), collagen type IV, and fibronectin, are assembled in a complex network known as the basement membrane (BM) which separates the epithelium from the stroma.[1] Ln-5, a heterotrimer glycoprotein formed by three disulfide-bonded subunits, α3, β3, and γ2,[1] regulates different cell functions such as adhesion, hemidesmosome formation, and migration.[2,3,4,5] It has been reported that Ln-5 is a major component of the BM, and its absence is responsible for lethal diseases.[6,7] Epithelial cells interact with Ln-5 via a family of transmembrane receptors, the integrins, which mediate cell adhesion as well as signal transduction.[8]

The BM regulates many cellular functions such as adhesion, migration, differentiation, and survival,[9] and its integrity is crucial to preserving epithelial architecture and organization.[10] The mammary gland represents an excellent model for studying interactions between the BM and epithelial cells.[11] At birth the mammary gland is histologically organized as a simple tree-like structure in which the ducts present only a few lateral branches.[12] As the duct tips cells are assembled in bulbous structures, the end buds specifically penetrate the surrounding stroma.[12] The epithelial cells located at the end tips are known as cap cells and are responsible for the elongation and the ramification of the ductal tree.[12] Mammary gland development and branching morphogenesis is controlled by sex steroids, as well as growth hormone, prolactin, and epidermal growth factor, each of which plays a mammogenetic role by stimulating epithelial cell proliferation and differentiation.[13,14] Also, other local factors, such as proteolytic remodeling of the ECM, are believed to be involved in mammary gland development and branching morphogenesis.[15,16] It has been reported that members of the matrix metalloprotease family, including stromely-

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sin-1 and matrix metalloprotease-2 (MMP2), are up-regulated in several conditions in which tissue remodeling occurs.[17] These enzymes are ubiquitously located in the body, are capable of degrading several proteins, including BM components,[10] and are considered to have a key role in tissue morphogenesis and in cancer metastasis as well.[10,18] In a transgenic mouse model, stromelysin-1 has been shown to induce branching morphogenesis[16,19] and also to trigger a malignant phenotype in epithelial mammary cells after extended contact.[20] While sex steroids regulate branching morphogenesis of the mammary gland, as is proteolytic ECM remodeling, no mechanistic link between these two processes has yet been established.

We have recently reported that human breast epithelial cells are able to migrate *in vitro* on MMP2-cleaved but not on intact Ln-5.[4] MMP2 cleaves the γ chain of Ln-5, generating an 80-kd fragment referred to as γ2x. This fragment is present in tumors and in some tissues undergoing remodeling but absent in quiescent tissues.[4] Together, these findings support a model whereby local secretion and/or activation of MMP2 in the proximity of the epithelial BM results in the cleavage of Ln-5 to promote epithelial cell migration. This model may be particularly suitable in mammary gland studies, since this organ undergoes dramatic remodeling after puberty.

The goal of this study was to investigate the cleavage of Ln-5 by MMP2 in breast tissue remodeling. Initially, using the γ2x fragment as a marker for MMP2 cleavage, we could show that its presence occurs exclusively during mammary gland tissue remodeling. We further showed that treatment with sex steroids can induce the cleavage of Ln-5 in the mammary gland of sexually immature rats and that epithelial reorganization and tissue remodeling occur in cultured explants of rat mammary gland where Ln-5 is cleaved by the addition of MMP2. Similar epithelial rearrangements and morphological changes were induced also in cells in contact with human Ln-5 after exposure to MMP2.

Materials and Methods

Induction of Mammary Gland Development in Vivo

Sexually immature (12 days old, less than 30 g of body weight) female Wistar rats were treated with a combination of sex steroids (estrogen and progesterone) (Sigma, St. Louis, MO) as previously described.[21] Briefly, hormones were dissolved in sesame oil and injected subcutaneously in animals at a dosage of 500 μg of estrogen once a day and 2 mg of progesterone twice a day. Control animals received vehicle alone. Animals were treated for 3 days and euthanized 24 hours after the last injection by CO_2 inhalation. Mammary glands were explanted and either immediately snap frozen in liquid nitrogen or imbedded in OCT.

All of the experiments were approved by and conformed to the guidelines of the Institutional Animal Care Committee.

Tissue Harvesting

Mammary gland tissue was collected from rats or mice less than 2 weeks of age for the sexually immature stage, at 8 weeks for the sexually mature stage, at 13.5 days of pregnancy, at 12 days after the onset of lactation, or at 8 days after weaning.

Western Blot Analyses

Tissues were pulverized, washed, and resuspended in sample buffer as previously described.[4] The total amount of protein was measured using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL) and normalized amount (300 μg of protein loaded into each lane) was separated by SDS-PAGE on a 6% polyacrylamide gel under reducing conditions and transferred to polyvinylidene difluoride membranes (BioRad, Hercules, CA) for western blot analyses, performed as described.[4]

Antibodies

Rabbit antiserum 1963, which recognizes the γ2 subunit of rat Ln-5, was prepared as described.[4] Rabbit polyclonal antiserum Ab45 to MMP2 was also described.[22] Antiserum 2794 was prepared by immunizing rabbits with a glutathione-*S*-transferase fusion protein prepared by cloning in the vector pGEX the coding region of the Ln-5 γ2 chain corresponding to the last 126 residues of the COOH terminus (residues 1046–1171).

Protease Activation

Human recombinant pro-MMP2[23] was activated with 1 mmol/L paraminophenylmercuric acetate in a buffer containing 50 mmol/L NaCl, 5 mmol/L $CaCl_2$, and BRIJ 35% 0.01%, pH 7.2, for 30 minutes at 37°C[24]. Human recombinant pro-MMP9[25] was incubated with the activating buffer for 3 hours at 45°C. Enzyme activation was verified by zymography.

Mammary Gland ex Vivo *Explants*

Mammary glands were explanted under sterile conditions, washed with phosphate-buffered saline (PBS), sliced into 1-mm cubes, cultured in DFCI medium without serum or in serum-free DMEM supplemented with L-glutamine and antibiotics, submerged on 24-transwell filters (Corning Costar, Cambridge, MA). Explants were incubated for 24 hours at 37°C in a CO_2 incubator in the presence of recombinant active MMP2 at concentrations of 30, 10, or 3 nmol/L, MMP9, or plasmin (Enzyme Research Laboratory, South Bend, IN). In some cases, the MMP inhibitor, BB94[26] (kindly provided by British Bio-Technology, Ltd), at concentrations of 500 nmol/L, was added.

Electron Scanning Microscopy, Histology, and Immunohistochemistry

Mammary gland explants were either paraffin-embedded, sectioned, and analyzed by scanning electron microscopy (stereoscan 360 scanning electron microscope at an accelerating voltage of 10 kV) or snap-frozen in liquid nitrogen, embedded in OCT compound (Miles Laboratories Inc., Naperville, IL), cut into 5-μm sections with a microtome (model HM 505E, Carl Zeiss, Oberkochen, Germany), and stained with antibodies.[27]

Whole-mount staining of mammary glands was performed as described.[12] Briefly, glands were flattened on a tissue capsule, fixed in Telly's fixative, defatted in three changes of acetone, hydrated in 95% ethanol, and stained with hematoxylin (0.65 g of $FeCl_3$, 67.5 ml of H_2O; 8.7 ml of 10% hematoxylin in 95% ethanol, pH 1.25). Glands were rinsed in water, destained in acid ethanol, dehydrated in increasing ethanol concentrations, indefinitely stored in methyl salicylate, and photographed using a Zeiss microscope.

Cell Cultures

MCF-10 cells, a spontaneously immortalized human breast epithelial cell line,[28] was maintained in culture in DFCI medium composed of a 1:1 mixture of modified Eagle's medium and Ham's F12 media (Gibco, Grand Island, NY), and enriched with 1% fetal bovine serum and growth factors.[29]

804G cells, derived from a rat urinary bladder carcinoma, were cultured in DMEM medium, supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, penicillin (20 U/ml), and streptomycin (20 mg/ml).

Deposited ECM Preparation

Deposited Ln-5 was prepared from the human cell lines MCF-10[30] or the rat 804G cell line.[31,32] Briefly, cells were cultured for 3 days to confluency and then removed according to described procedures[33] that leave behind functional ECM. The ECM from the above cells is known to be highly enriched in Ln-5.[30,34]

Cell Morphology Assays

Glass coverslips were coated with Ln-5-enriched ECM by cell deposition, as described above. In some cases, glass coverslips were coated with ECM secreted by the cell line 804G by incubating them in 804G conditioned medium overnight. For Ln-5 depletion, the 804G medium was passed through an anti-Ln-5 TR1 antibody affinity column before incubation with coverslips. Coated coverslips were treated with MMP (130 nmol/L in DMEM) or control medium as described above. MCF-10 cells (150,000 cells per coverslip) were incubated for 2 hours on the coverslips in serum-free medium in a humidified CO_2 incubator at 37°C. Unattached cells were gently removed with PBS, and the remaining attached cells were fixed with 3% paraformaldehyde in PBS.

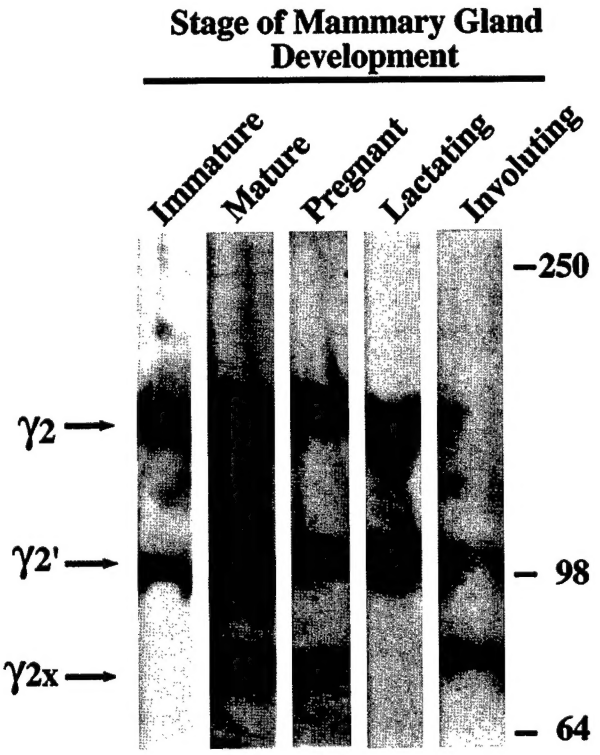


Figure 1. Cleaved Ln-5 is present in remodeling but not in quiescent mammary gland. Rat mammary glands were collected at different stages of maturation as indicated. Tissues were pulverized and examined by western blot analysis for the presence of Ln-5. The γ2 subunit migrates as a 135-kd band and a 100-kd band (γ2'). The latter is a biosynthetic maturation product of the 135-kd form, which occurs by proteolysis of the amino terminus.[1] The γ2x band at 80 kd was used to indicate the presence of MMP2-cleaved Ln-5. The γ2x band was absent in sexually immature (less than 2 weeks old) rat but was present in the sexually mature (8 weeks old) rat, as well as during pregnancy. During lactation γ2x was absent, but it reappeared during involution. Tissue of involuting mammary gland is from mice.

Cells were photographed with phase-contrast optics on a Zeiss Axiophot microscope. Cell areas were measured using a Bio-Rad MRC600 confocal microscope and CoMOS software, which calculates areas based on manual outlining of individual cells. Typically, areas of 80 cells were measured and averaged.

Results

Cleaved Ln-5 Is Present Only in Remodeling Mammary Gland

To determine whether cleavage of Ln-5 is associated with tissue remodeling, we examined by Western blot analysis rat mammary gland tissue at various stages of sexual maturation for the presence of γ2x, an 80-kd proteolytic fragment of Ln-5 generated by MMP2 digestion.[4] As shown in Figure 1, γ2x was present in sexually mature, pregnant, and involuting (ie, postlactating) rat mammary gland tissue. In contrast, the γ2x fragment was not detected in sexually immature or in lactating animals. Therefore, the presence of γ2x correlates well with stages of active remodeling of the gland, both when the duct network is actively expanding (ie, during sexual maturation

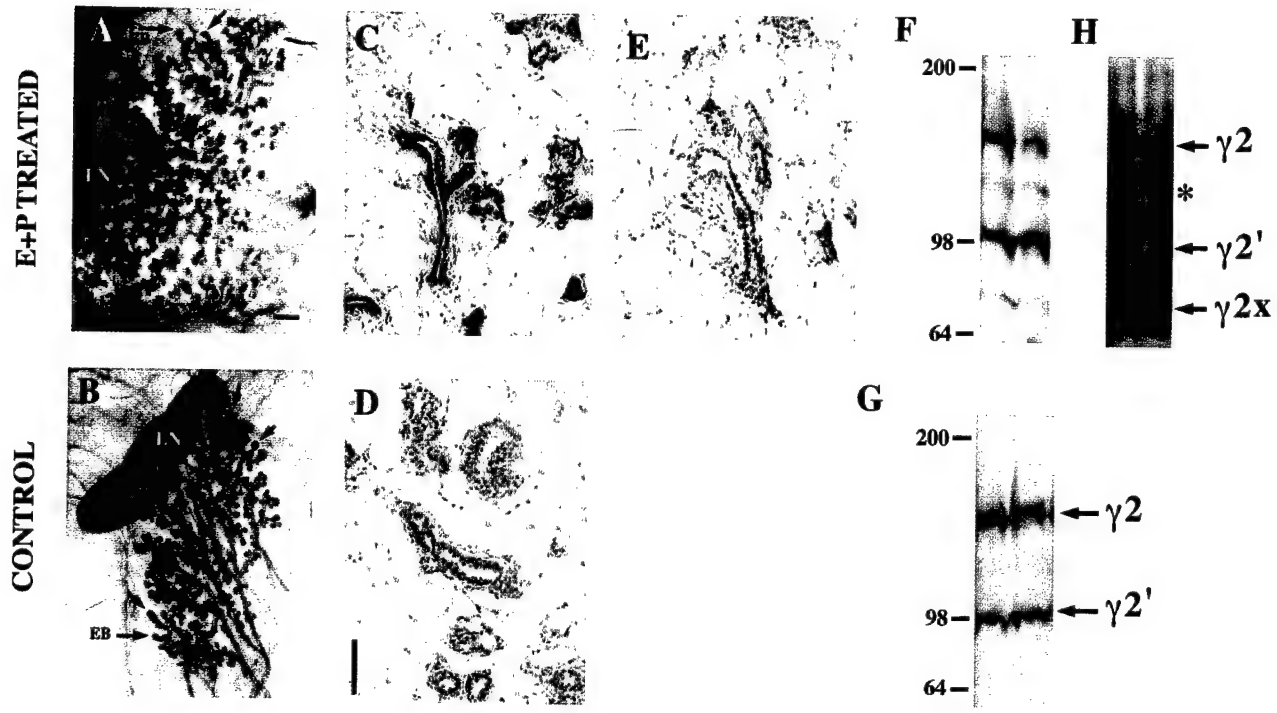


Figure 2. MMP2 and cleaved Ln-5 are present in remodeling mammary glands from sexually immature rats treated with estrogen (E) and progesterone (P). Animals were treated with sex steroids (E+P) or vehicle (control) as described under Materials and Methods. Increased arborization of the mammary gland was observed in hormone-treated compared to control animals (A, B) by whole-mount mammary glands. Frozen sections were immunostained with a MMP2 polyclonal antiserum (C, D) or as a control with secondary antibody alone (E). Western blots showed uncleaved Ln-5 in tissue extracts from control animals and cleaved Ln-5 in hormone-treated animals. (Each lane represents a different animal.) The mapping of the γ2x fragment by using the polyclonal antiserum 2794 (H) demonstrates that this fragment is the same fragment we found and investigated previously *in vitro*.[4] The band at 120 kd (*) was consistently observed with both antisera in the hormone-treated animals, but its nature is unknown. Scale bar, 0.05 mm (A); 100 μm (C, D, E).

and pregnancy) and the end buds are invading the stroma, and when they are involuting (ie, postweaning) and reduction of branches and alveoli occurs.

Sex Steroids Induce MMP2 Expression and Cleaved Ln-5 in Remodeling Mammary Gland Tissue

To determine whether sex hormones may induce ECM proteolysis in the mammary gland, we injected sexually immature female rats with estrogen and progesterone.

The results shown in Figure 2 are representative of four identically treated animals. As expected, mammary glands in hormone-treated rats (Figure 2A) were more developed than in control rats (Figure 2B). Several new side branches in the ductual arborization were observed, and new end buds with a larger diameter penetrated extensively the fat pad tissue. The glandular parenchyma of treated glands occupied a larger volume in the fat pad than controls (Figure 2).

Immunohistochemical analysis revealed that MMP2 was readily detectable in mammary gland specimens from hormone-treated animals (Figure 2C), while it was completely absent in untreated animals (Figure 2D). The staining was localized at the periphery of the lobules in the BM zone, mostly concentrated around the myoepithelial cells, and it was occasionally present in some luminal cells. Some stromal cells also showed positive MMP2 staining. In adjacent sections, the secondary antibody alone (Figure 2E) showed no staining, indicating specificity of the MMP2 staining.

In the same rats, the presence of Ln-5 in mammary tissue samples from hormone-treated and control animals was investigated by Western blot analysis. In the sexually immature untreated rats, Ln-5 was present in the intact form (Figure 2F). In contrast, in the hormone-treated animals the γ2x chain (Figure 2, G and H) was easily detectable, indicating the presence of cleaved Ln-5. The polyclonal antiserum 2794 directed against the carboxyl terminal end of the Ln-5 γ chain was used to confirm the identification of the γ2x fragment in the E+P treated animals (Figure 2H).

These data suggest that sex steroids may induce MMP2 cleavage of Ln-5 in remodeling mammary tissue.

MMP2-Cleaved Ln-5 Induces Mammary Epithelial Reorganization in an ex Vivo *Mammary Gland Culture*

To better visualize the effects of MMP2 on the epithelial architecture of the mammary gland, we developed an *ex vivo* assay in which fragments of explanted mammary glands were maintained in culture in the presence of MMP2, MMP9, plasmin, or control medium. Histological and scanning microscopic analyses revealed dramatic changes in the epithelial organization of samples incubated with MMP2 (Figure 3B). No substantial changes were detected, however, in control medium specimens

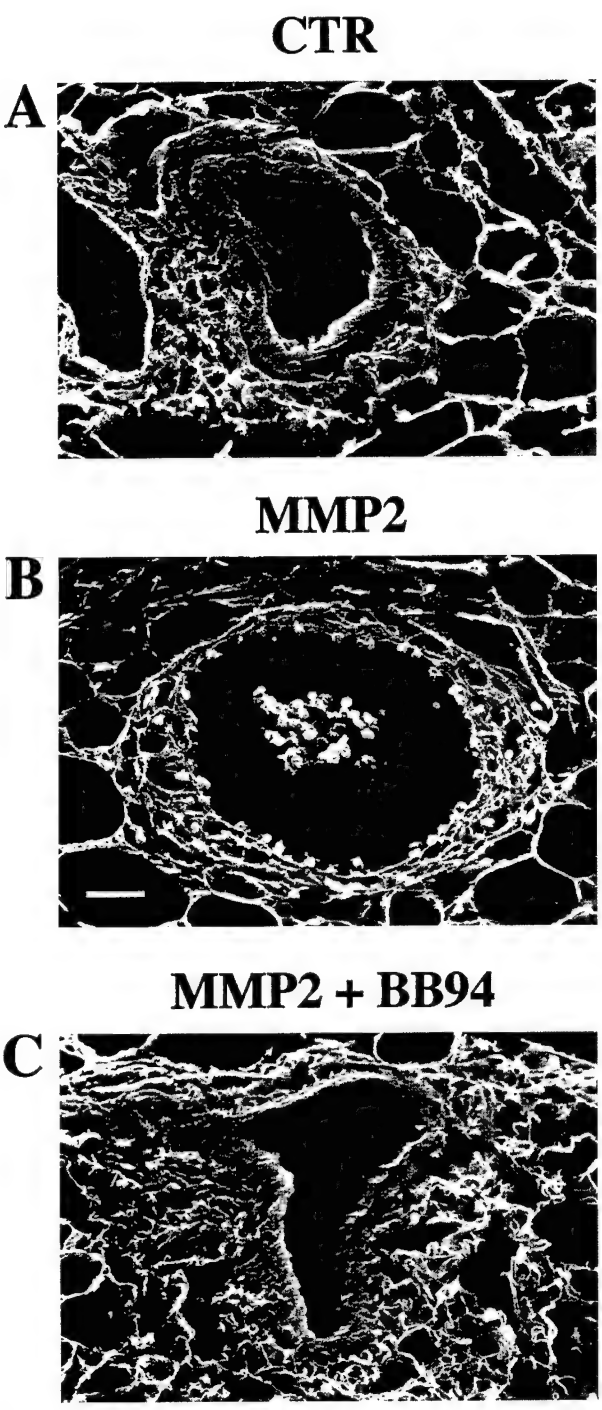


Figure 3. MMP2 alters the cellular architecture of the breast epithelium. Explanted mammary glands from sexually immature rodents were cultured *in vitro* (see under Materials and Methods). In control samples (A), the cells of the mammary epithelium were in close contact with each other showing typical quiescent epithelial organization. In the presence of MMP2 (B), some of the cells from the epithelial breast sheet lost contact with the BM and gathered in the center of the lumen, while other cells still in contact with the BM had a dramatically altered morphology. They had lost contact with each other and were rounded or elongated in shape. This effect was blocked by BB94, an inhibitor of MMPs (C). Scale bar, 18 μm.

(Figure 3A) or in specimens treated with MMP9 or plasmin (data not shown). In the MMP2-treated explants, some luminal epithelial cells were detached from the BM and grouped within the lumen of the mammary ducts.

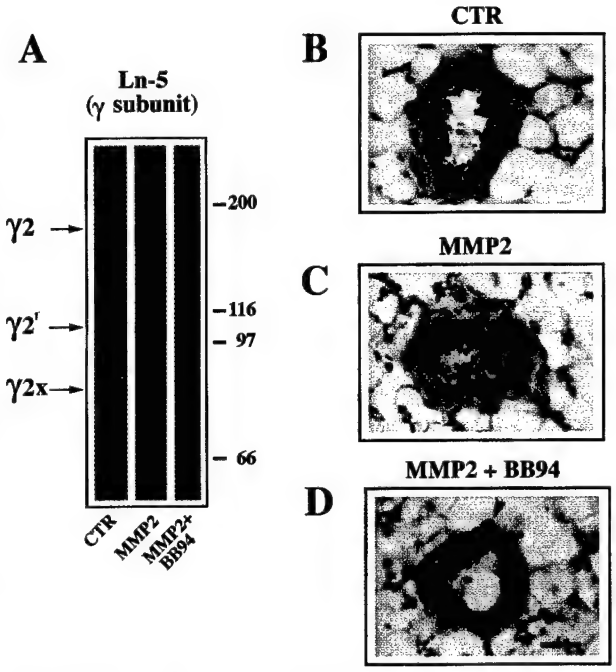


Figure 4. MMP2 cleaves Ln-5 in explanted mammary gland tissue but does not dissolve the BM. Cultured fragments of mammary glands from sexually immature rats (Figure 3) were processed for either Western blot analysis (A) or immunohistochemistry (B). Western blot analysis (A) using polyclonal antibody 1963, which recognizes the γ chain of Ln-5, revealed the presence of the 80-kd γ2x fragment band in cultures treated with MMP2 (C) but not in the control cultures (B) or in the cultures co-incubated with BB94 (D). By immunohistochemistry, Ln-5 was localized evenly along the BM in the samples treated with MMP2 (B) as well as in the control cultures (A) and those co-treated with MMP2 and BB94 (C). Scale bar, 60 μm.

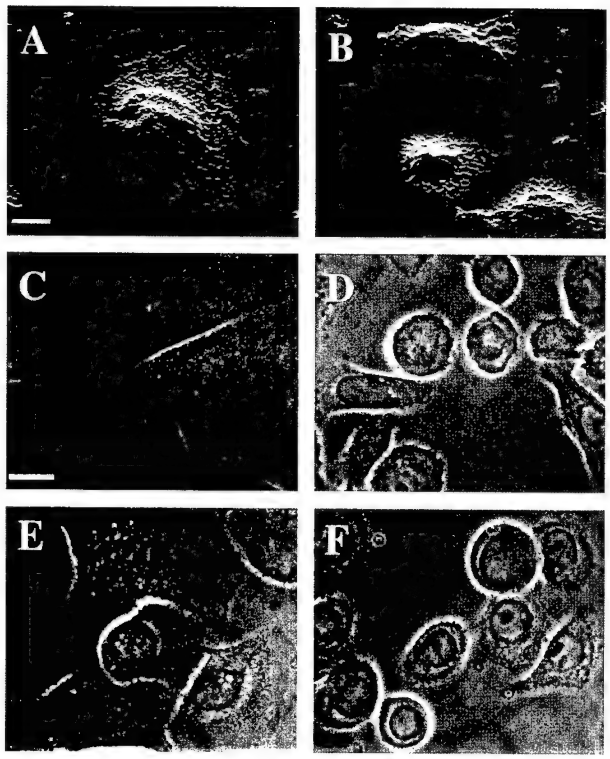
Moreover, cells still in contact with the BM had changed their morphology, some rounder and others more elongated compared to the control samples. This effect of MMP2 was dose-dependent and was completely inhibited by the presence of the MMP inhibitor BB94 (Figure 3C).

Tissues were processed in parallel for Western blot analysis and examined for the presence of the γ2x proteolytic fragment. As shown in Figure 4A, γ2x was detected only in those specimens treated with MMP2 and not in control tissue or in tissue cultured with MMP2 and BB94.

We also localized Ln-5 in the BM of the MMP2-treated mammary gland explants immunohistochemically using a polyclonal antibody to the γ subunit of Ln-5 (Figure 4B). Ln-5 staining was evenly distributed in the BM of ducts and alveoli, where it appeared as a continuous ring in control and in MMP2-treated tissue. This observation suggests that cleaved Ln-5 remains localized in the BM, that is, in the vicinity of the epithelial cells and is not removed by MMP2 digestion. Taken together, these results indicate that exogenously applied MMP2 cleaves Ln-5 *in situ*, resulting in a rearrangement of the epithelial organization of the mammary gland.

MMP2-Treated Human Ln-5 Induces Epithelial Reorganization in Vitro

To extend these findings to the human breast gland, we investigated the epithelial reorganization induced by



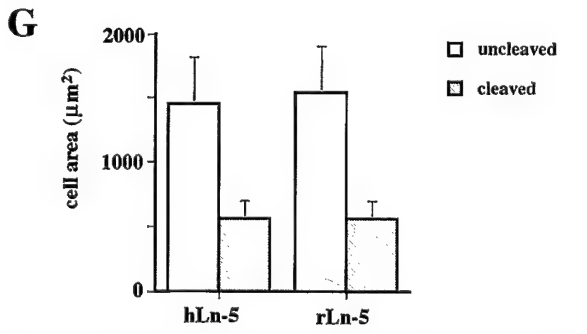


Figure 5. Changes in morphology of MCF-10 cells on MMP2-cleaved Ln-5. By scanning electron microscopy, MCF-10 cells plated on uncleaved human Ln-5 (A) appeared flat and large, a morphology typical of an immobile cell. In contrast, cells grown on cleaved human Ln-5 (B) were either elongated with filopodia and lamellipodia or rounded, having the appearance of a motile phenotype. MCF-10 cells were plated on rat (C, D) or human (E, F) Ln-5, either cleaved or uncleaved, for 90 minutes. MCF-10 cells were large and in contact with each other on uncleaved rat or human Ln-5 (C, E), where they assumed a typical epithelial-like polygonal shape. In contrast, cells on MMP2-cleaved rat and human Ln-5 (D, F) had lost contact with each other and appeared round or elongated with a ruffling in the membrane. The difference in cell size was quantified by measuring the cell surface area using a computerized analysis system. The cell surface area was similar whether MCF-10 cells were cultured on human or rat Ln-5 (G), but there was a large reduction in cell size in cultures grown on cleaved human or rat Ln-5. This size difference reflects the morphological changes we observed and suggests that rat and human Ln-5 have similar functional roles in epithelial reorganization. Scale bars, 6 μm (A, B) and 10 μm (B, D, E, F).

MMP2 in a normal human mammary epithelial cell line, MCF-10, which secretes and deposits Ln-5[30] and which under appropriate culture conditions grows as an organized epithelial monolayer

MCF-10 cells plated on human Ln-5 appeared flattened (Figure 5, A and E), while on MMP2-treated Ln-5 they assumed a morphology typical of a motile phenotype (Figure 5, B and F). The changes in morphology observed were similar to those seen on MMP2-treated rat Ln-5 (Figure 5, C and D). Cells plated on either rat or human Ln-5 were large, organized in close contact with each other, and formed epithelium-like structures (Figure 5, C and E). In contrast, cells plated on MMP2-treated human or rat Ln-5 (Figure 5, D and F) were smaller, some rounder, others more elongated with filopodia and lamellipodia. Overall, cells plated on either human or rat intact Ln-5 had an average cell area three-fold greater than those plated on cleaved Ln-5 (Figure 5G).

To test whether or not MMP2-induced morphology changes were dependent on Ln-5, glass coverslips were coated with 804G spent medium, which contains soluble Ln-5, and, in parallel, with 804G medium depleted of Ln-5 with a specific antibody (see under Materials and Methods). Within 2 hours, cells were completely spread on coverslips coated with spent medium (Figure 6A) but not with depleted medium (Figure 6C), indicating that Ln-5 had been removed by the depletion procedures. Furthermore, on addition of MMP2, a change in morphology, with appearance of filopodia and cell polarization, was observed in coverslips coated with Ln-5 containing medium, but not with the depleted medium (Figure 6, B and D). Those results suggest that MMP2 induces changes in morphology only in the presence of Ln-5.

Discussion

We present evidence that proteolytic cleavage of Ln-5 by MMP2 occurs during the remodeling of breast gland tissue induced by sex steroids. To summarize: 1) In mammary gland tissue, Ln-5 was consistently cleaved during stages of remodeling (eg, in sexually mature, pregnant, and involuting rats), but was intact in quiescent tissue (eg, before puberty or during lactation). 2) Ln-5 was cleaved and MMP2 was detectable during mammary gland expansion induced by the injection of sex steroids in sexually immature rats. 3) In *ex vivo* assays, the mammary epithelium was rearranged and Ln-5 was cleaved when mammary gland specimens were incubated with MMP2. 4) The remodeling of the epithelial architecture induced by MMP2 was dose-dependent and protease-specific, since no effect was observed with other proteases (eg, MMP9 or plasmin. 5) Finally, morphological changes observed in the presence of cleaved Ln-5 were reproducible in a human breast cell line, MCF-10, *in vitro*. Together, our data suggest an interaction between MMP2 and Ln-5 may occur during the remodeling of the mammary gland induced by sex steroids.

The ECM likely plays an important role in tissue development and differentiation. For instance, specific differentiated functions of mammary cells[9] are supported by contact with the ECM. A role for the ECM in morphogenesis has also been proposed and is supported by some experimental evidence.[35] Epithelial-mesenchymal interactions, mediated at least in part at the BM interface, are critical in regulating branching morphogenesis.[15,16] Interestingly, the structure of the BM may vary in the mammary gland. At quiescent sites, next to end buds, the BM

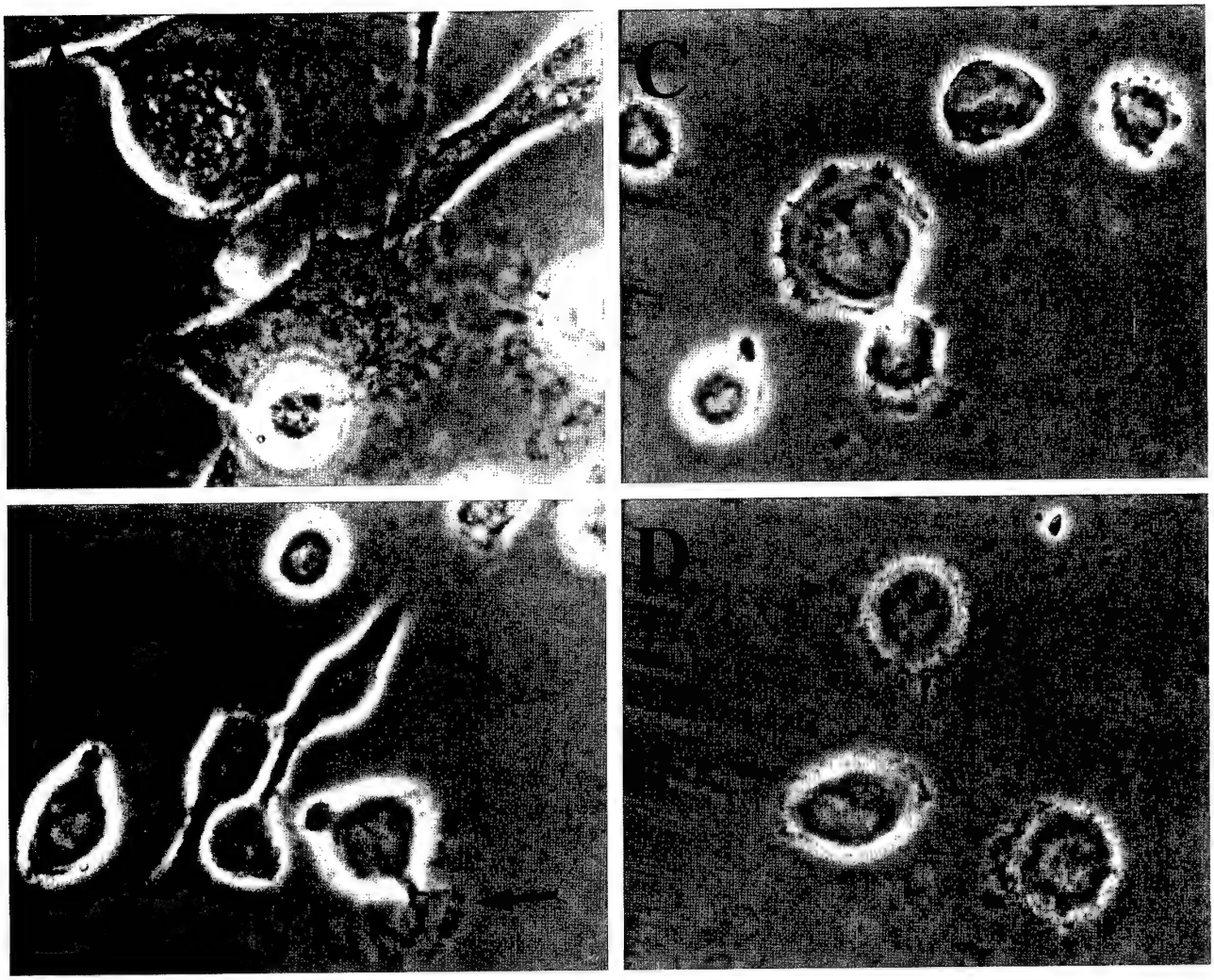


Figure 6. MCF-10 cell morphology is dependent on the presence of Ln-5 and cleaved Ln-5. MCF-10 cells plated on coverslips coated with Ln-5-enriched ECM (**A**) appear well spread, while they are still poorly spread on coverslips coated with ECM depleted of Ln-5 by absorption with a specific antibody (**C**). MCF-10 cells become elongated in the presence of MMP2 on Ln-5 enriched ECM (**B**) but not on Ln-5 depleted ECM (**D**). Scale bar, 10 μm.

is 14-fold thicker than at the tip of end buds, where actively branching occurs or invading epithelial cap cells are located.[12] These cells penetrate into the fat tissue until they completely fill it.[12] Our results show that an important component of the BM, Ln-5, is proteolitically cleaved during remodeling of the mammary gland, and that its cleavage correlates with reorganization of the gland induced by sex steroid treatment. It remains to be determined whether Ln-5 cleavage plays a direct role in the subsequent reorganization of the gland.

Consistent with other observations,[36] in our experiments MMP2 was detected immunohistochemically in stromal cells and occasionally in luminal cells, but it was mostly present in the cytoplasm or in the proximity of the myoepithelial cells. This is the site where new branches are generated[37] and where ECM remodeling occurs.[36,38] It is not known, however, how ECM remodeling affects cell behavior. Our data suggest that the cleavage of Ln-5 may represent a structural change in the ECM that causes induction and guidance of cell migration during tissue reorganization. Since cultured cells plated on Ln-5 alone can be induced to migrate in the presence of MMP2, the presence of MMP2-cleaved Ln-5 may itself be sufficient for migration.[4] We cannot rule out, however, that additional mechanisms for MMP2-induced remodeling exist, or that proteases other than MMP2, such as MMP3, may cleave Ln-5 to produce a migratory substrate. More studies are necessary to clarify these points.

MMP2 and cleaved Ln-5 were detected both during branching morphogenesis and involuting phases of breast gland remodeling. Additional studies are necessary to pinpoint the exact locations of MMP2 and cleaved Ln-5 in the mammary gland tree with respect to areas of branching or reductive morphogenesis. Unfortunately, at this time, we do not have reagents that can distinguish between intact and cleaved Ln-5, which would be critical for such studies.

Our experiments with tissue explants indicate that although MMP2 can have a disruptive effect on mammary epithelial architecture, it does not degrade the BM or affect other tissue types. The changes we observed, however, do not resemble branching morphogenesis, which presumably requires several additional signals in a coordinated fashion. Nevertheless, even though it does

not faithfully reproduce the *in vivo* process, the tissue explant technique may be useful to test factors that affect epithelial organization and dissect their mechanism of action.

How mammary epithelial cells acquire motility on MMP2-cleaved Ln-5 is not yet known; however, it is possible that integrins might play a role in interpreting ECM cues. Both $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins can interact with Ln-5.[39] Yet while $\alpha_3\beta_1$ integrins are involved in adhesion and migration via focal adhesions, $\alpha_6\beta_4$ supports the formation of hemidesmosomes which link the intermediate filament cytoskeleton to the BM.[40] It is likely that these integrins are affected differentially by MMP2 actions on Ln-5. For instance, on cleaved Ln-5 it is expected that the anchoring functions of hemidesmosomes may be lost, whereas the migratory functions of $\alpha_3\beta_1$ may be enhanced. In addition, we cannot rule out the possibility that also $\alpha_6\beta_4$ may have a role on cleaved Ln-5, since it has been reported that it is involved in migration.[41] These issues remain to be to be elucidated at the molecular level.

At the functional level, both rat and human Ln-5 behaved similarly. That is, on treatment with MMP2 they supported changes in cell morphology. However, we have been unable to characterize the structural consequences of MMP2 treatment on Ln-5 because of the scarcity of that material.

In a more general sense, our results point to the possibility that, during breast branching morphogenesis, proteases may unveil ECM cues that guide the morphogenetic process itself. Their role, then, may be more intricate than simple degradation of ECM barriers. Direct links still remain to be established, however, between sites of ECM proteolysis and the presence of putative morphogenetic cues. Other factors, such as sex hormones, may also directly or indirectly activate ECM proteolysis and *vice versa*, ECM proteolysis might influence cellular responsiveness to sex steroids, eg, via integrin-mediated signaling.[42] Model systems, such as the *ex vivo* explants we describe here, may help discover the molecular mechanism underlying the complex events of tissue remodeling.

Acknowledgments

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Role of Cell Surface Metalloprotease MT1-MMP in Epithelial Cell Migration over Laminin-5

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Abstract. Laminin-5 (Ln-5) is an extracellular matrix substrate for cell adhesion and migration, which is found in many epithelial basement membranes. Mechanisms eliciting migration on Ln-5 need to be elucidated because of their relevance to tissue remodeling and cancer metastasis. We showed that exogenous addition of activated matrix metalloprotease (MMP) 2 stimulates migration onto Ln-5 in breast epithelial cells via cleavage of the γ2 subunit. To investigate the biological scope of this proteolytic mechanism, we tested a panel of cells, including colon and breast carcinomas, hepatomas, and immortalized hepatocytes, selected because they migrated or scattered constitutively in the presence of Ln-5. We found that constitutive migration was inhibited by BB94 or TIMPs, known inhibitors of MMPs. Limited profiling by gelatin zymography and Western blotting indicated that the ability to constitutively migrate on Ln-5 correlated with expression of plasma membrane bound MT1-MMP metalloprotease, rather than secretion of MMP2, since MMP2 was not produced by three cell lines (one breast and two colon carcinomas) that constitutively migrated on Ln-5. Moreover, migration on Ln-5 was reduced by MT1-MMP antisense oligonucleotides both in MMP2+ and MMP2− cell lines. MT1-MMP directly cleaved Ln-5, with a pattern similar to that of MMP2. The hemopexin-like domain of MMP2, which interferes with MMP2 activation, reduced Ln-5 migration in MT1-MMP+, MMP2+ cells, but not in MT1-MMP+, MMP2− cells. These results suggest a model whereby expression of MT1-MMP is the primary trigger for migration over Ln-5, whereas MMP2, which is activated by MT1-MMP, may play an ancillary role, perhaps by amplifying the MT1-MMP effects. Codistribution of MT1-MMP with Ln-5 in colon and breast cancer tissue specimens suggested a role for this mechanism in invasion. Thus, Ln-5 cleavage by MMPs may be a widespread mechanism that triggers migration in cells contacting epithelial basement membranes.

Key words: migration • extracellular matrix • epithelial cell • invasion

Introduction

Cell motility is a determinant of epithelial morphogenesis and regeneration (Thiery, 1984). An important issue is to define the molecular nature of spatial cues in the environment surrounding epithelial cells, which may signal initiation of migration during processes such as tissue remodeling or wound healing. Finding and characterizing these cues should make it possible to understand and manipulate epithelial tissue organization and pathological conditions such as metastasis.

The extracellular matrix (ECM)[1] of the basement membranes (BMs) is a likely structural site for motility cues since the BM is a critical interface between epithelial cells and the rest of the body. Laminin-5 (Ln-5), an ECM glycoprotein found in the BM, is a strong candidate for playing a major role in epithelial cell motility (Miyazaki et al., 1993b; Giannelli et al., 1997). We hypothesized that it may act not only as a passive ECM substrate (Roskelley et al., 1995; Malinda and Kleinman, 1996), but may actively participate in the regulatory aspects of motility.

Ln-5 is a recognized ligand for integrins α6β4 and α3β1 (Carter et al., 1990; Jones et al., 1991; Niessen et al., 1994;

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[1]*Abbreviations used in this paper:* AS, antisense oligonucleotide; BM, basement membrane; CM, conditioned medium; ECM, extracellular matrix; HLD, hemopexin-like domain; Ln-5, laminin-5; MMP, matrix metalloprotease; MT1-MMP, membrane type 1-MMP; TIMP, tissue inhibitor of metalloprotease.

Ryan et al., 1994). The interaction of Ln-5 with α6β4 leads to the assembly of hemidesmosomes, which are static adhesive devices that anchor epithelial cells to the underlying BM (Baker et al., 1996). Interestingly, though, Ln-5 was also shown to promote vigorous cell scattering when added to the medium of epithelial cell cultures (Miyazaki et al., 1993b; Giannelli et al., 1997). These apparently opposing functions of Ln-5 (i.e., the ability to induce either static adhesion via hemidesmosomes [Jones et al., 1991] or cell motility [Miyazaki et al., 1993b; Giannelli et al., 1997]) may reflect physiological mechanisms to maintain tissue integrity: in quiescent tissues, Ln-5 may be predominantly a static adhesive substrate, whereas during regenerative or wound healing responses, it may deliver migratory stimuli. In support of this hypothesis, primary breast epithelial cell cultures, and the immortalized breast cell line MCF-10A, become migratory on Ln-5 upon addition of activated MMP2, an ECM metalloprotease (Giannelli et al., 1997). An indication that this mechanism may have physiological significance is that Ln-5 γ2 fragments, corresponding in size to those generated by MMP2 cleavage, are detectable in remodeling, but not in quiescent mammary glands (Giannelli et al., 1999) and in epithelial tumor tissue specimens. Thus, MMP2 cleavage of Ln-5 may play a mechanistic role in epithelial cell invasion during tissue remodeling as well as in cancer invasion and metastasis. However, these processes require some degree of spatial definition. Since MMP2 is secreted in the extracellular space and it is soluble, it is not clear how Ln-5 cleavage by MMP2 may be spatially directed to discrete BM sites.

Several MMPs have been associated with the remodeling of epithelial tissues as well as metastasis (Birkedal-Hansen, 1995), but the molecular mechanisms remain poorly understood (Werb et al., 1996). One critical issue is MMP activation and its spatial restrictions. In the breast epithelial cells we studied (primary cultures and MCF-10A), exogenous addition of chemically activated MMP2 was an absolute requirement for Ln-5 cleavage and consequent migration, raising the possibility of alternative, though not mutually exclusive, scenarios including the following: (1) epithelial cells may rely on another cell, e.g., mesenchymal or inflammatory, to activate pro-MMP2, cleave Ln-5, and migrate; (2) in cell cultures, MMP2 mimics the activity of other physiological proteases, which may be secreted and/or activated by epithelial cells; and (3) epithelial cells, upon responding to appropriate stimuli, may secrete MMP2, express the MMP2 activation apparatus, and thereby regulate migration on Ln-5 in an autonomous fashion.

To investigate these possibilities, we characterized the role of MMP during Ln-5 migration in a panel of cell lines of distinct histologic derivation. These cell lines present one critical difference with respect to MCF-10A and primary mammary epithelial cells: they do not require exogenous addition and/or activation of MMPs, but rather they migrate on Ln-5 constitutively. Here, we show that this constitutive Ln-5 migration depends upon expression of surface membrane type 1 (MT1)–MMP, which can both directly cleave Ln-5 and activate MMP2, if present, for further cleavage and migration. These data indicate that the MMP cleavage mechanism for induction of migration over Ln-5 may be of general importance in epithelial cells contacting the BM. Furthermore, because MT1-MMP is cell surface anchored, they provide a mechanism for spatially defined formation of promigratory cues along the BM.

Materials and Methods

Cell Lines and Culture Conditions

BRL is a nontumorigenic liver epithelial cell line from Buffalo rat (Nissley et al., 1977). MCF-10A is a spontaneously immortalized human mammary epithelium cell line, and 804G is a rat bladder carcinoma. Types of human cancer cell lines are as follows: MDA-MB-231, breast carcinoma; DLD-1 and HT-29, colon carcinoma; HLE and HLF, hepatoma; and HT-1080, fibrosarcoma. BRL, MCF-10A, MDA-MB-231, DLD-1, HT29, and HT-1080 cell lines were obtained from American Type Culture Collection. HLE and HLF were obtained from the Japanese Cancer Resources Bank. The 804G cell line was described previously (Falk-Marzillier et al., 1998). Each cell line, except for MCF-10A, was maintained in DME (GIBCO BRL) plus 10% (vol/vol) FCS (Irvine Scientific), penicillin, and streptomycin. MCF-10A was maintained in DFC-1 plus 1% (vol/vol) FCS as described previously (Plopper et al., 1998).

Purification of Rat Ln-5 from 804G Serum-free Conditioned Medium (CM)

Serum-free DME CM of 804G cells was prepared in roller bottles, concentrated ~100-fold by ammonium sulfate at 80% saturation and dialyzed against 20 mM Tris-HCl (pH 7.5)/0.5M NaCl/0.005% Brij-35 (TNB buffer). Nonfunctional Ln-5 mouse mAb TR-1 (Plopper et al., 1996) was chemically conjugated to protein A–Sepharose 4B as previously reported (Koshikawa et al., 1992). Concentrated CM was passed over the TR-1 antibody column (0.8 × 4.0 cm; Bio-Rad Laboratories), previously equilibrated with TNB buffer at a flow rate of 15 ml/h. After washing with TNB, absorbed Ln-5 was eluted with 10 ml of 0.05% trifluoroacetic acid (TFA), pH 2.5. Each eluted fraction (1 ml) was quickly neutralized by 300 μl of 1 M Tris-HCl, pH 8.0, and then 1% of CHAPS (wt/vol) was added to each fraction.

Gelatin Zymography and Immunoblotting

Gelatin zymography was performed as reported (Koshikawa et al., 1992). Serum-free CM was prepared from confluent cultures of each cell line incubated for 48 h in serum-free basal media. The CMs were concentrated ~30-fold by ammonium sulfate at 80% saturation and dialyzed against TNB buffer (Koshikawa et al., 1992). Crude plasma membranes were prepared from confluent cultures of each cell line incubated for 48 h in serum-free basal medium. Cells were scraped in 0.25 M sucrose/10 mM Hepes, pH.7.5, and then collected in Eppendorf tubes. The cell suspension was homogenized at 4°C, nuclei removed by centrifugation at 3,000 rpm for 5 min, the supernatant was centrifuged at 15,000 rpm for 30 min, and the crude plasma membrane fraction was recovered as pellets. Immunoblotting was performed with rabbit polyclonal antibodies against human membrane type 1 MMP (MT1-MMP) and mouse mAb against human tissue inhibitor of metalloprotease-2 (TIMP-2) by reported methods (Miyazaki et al., 1993a), except that the antigen was detected by the enhanced chemiluminescence method with a NEN Life Science Products kit.

Preparation of MMP2 and MMP Inhibitors

Human TIMP-2–free MMP2 was purified from serum-free CM of human glioblastoma T98G cells (Miyazaki et al., 1993a). TIMP-1 and TIMP-2 were purified from serum-free CM of human bladder carcinoma EJ-1 cells (Miyazaki et al., 1993a). Hemopexin-like domain (HLD) of MMP2 was purified from human TIMP-2–free MMP2 treated with neutrophil elastase, by Reactive-Red agarose affinity chromatography as described before (Strongin et al., 1993; Rice and Banda, 1995). BB94 (Batimastat) was a gift from British Biotechnology Ltd.

Bacterial Expression and Purification of Recombinant Rat MT1-MMP

Rat smooth muscle cell–derived MT1-MMP cDNA encoding amino acids Ile^{114}-Glu^{528} was amplified by a PCR using 5′- and 3′-primers with addi-

tional NdeI and EcoRI sites at the ends, respectively. The PCR products were digested with these enzymes and subcloned into the pET-30a vector (Novagen, Inc.), modified to express the FLAG peptide (Kinoshita et al., 1998) fused to the COOH terminus of rat MT1-MMP protein in *Escherichia coli.* Other experimental methods were performed as previously reported (Kinoshita et al., 1998).

Cleavage of Ln-5 by MMP2 or MT1-MMP

5 μg of purified Ln-5 was incubated with 3.2 μg/ml of p-aminophenyl mercuric acetate–activated MMP2 for 2 h at 37°C in 50 mM Tris, pH 7.5, 0.005% Brij-35, 10 mM $CaCl_2$. In some cases, purified Ln-5 (1 μg) was adsorbed onto a 96-well plate well, and then incubated with recombinant MT1-MMP (0.2–2 μg/ml) for 16 h at 37°C in 50 mM Tris, pH 7.5, 0.005% Brij-35, 10 mM $CaCl_2$. After incubation, each reaction mixture was electrophoresed on 6% SDS-PAGE under reducing conditions, and then analyzed by Western blotting with a rabbit polyclonal antibody against rat Ln-5 γ2 chain antibody (1963).

Cell Scattering Assay

BRL cells were placed into 24-well plates containing 0.5 ml of DME plus 1% FCS at 7,000 cells per well. Purified Ln-5 (80 ng/ml) was added. After 6–24 h, cells were fixed in 100% methanol for 10 min and stained with 0.5% crystal violet/20% methanol. Scattering was judged by microscopic observation.

Cell Migration Assay

Cell migration assays were performed in Transwell chambers as reported (Mizushima et al., 1997). Cells were resuspended in DME plus 0.1% (wt/vol) BSA and seeded at 20,000 cells/well for BRL, MDA-MB-231, DLD-1, and HT-29; 10,000 cells for HLF; and 5,000 cells for HLE. Ln-5 was added to the lower chamber at 400 ng/ml (cancer cells) or 200 ng/ml (BRL). In some cases, MMP2-cleaved, MMP inhibitors, antibodies, HLD of MMP2, or oligonucleotides were also added, at indicated concentrations. After incubation (16 h for BRL, DLD-1, and HT-29; 6 h for MDA-MB-231 and HLE; 3.5 h for HLF), cells that migrated onto the lower surface of the filters were stained with 0.5% crystal violet/20% methanol and counted (Giannelli et al., 1997).

Antisense Oligonucleotides

Rat and human MT1-MMP antisense (AS) oligonucleotide sequences and their scrambled control oligonucleotides were designed by a computer program (Advanced Gene Computing Technologies). The following phosphorothioate oligodeoxyribonucleotides were synthesized: AS oligonucleotide, 5′-TCGTGTCCGAATCGG-3′; control oligonucleotide, 5′-TCCGAGTTCTCGAGG-3′ for rat MT1-MMP, or AS oligonucleotide, 5′-GCCGTAAAACTTCTG-3′; and control oligonucleotide, 5′-ATCTCGGATCAGACT-3′ for human MT1-MMP. These oligonucleotides were freshly dissolved in PBS and added to BRL or HT-29 cells, respectively, at 10 μM. After 2 d of pretreatment with test or control oligonucleotides, cells were tested in migration assays. Oligonucleotides were added to the lower chamber of Transwells at 10 μM.

Confocal Microscopy

Double immunofluorescence and confocal microscopic analyses were performed on 8-mm cryostat sections of human colon and breast carcinomas (The Cooperative Tissue Network), fixed in freshly made 4% formaldehyde (from paraformaldehyde) for 20 min at 4°C, permeabilized in 0.1% Triton X-100 for 10 min at room temperature, and then incubated in 50 mM glycine in PBS to saturate reactive groups generated by formaldehyde fixation. Nonspecific binding was blocked by incubation in PBS containing 2% donkey serum (DS) (Jackson ImmunoResearch Laboratories, Inc.) and 1% BSA (Sigma Chemical Co.) for 1 h at room temperature. After extensive washes in PBS-DS (0.2% donkey serum, 0.1% BSA, 5 mM glycine), sections were incubated for 1 h at room temperature with a mixture of primary antibodies (5 μg/ml), or a mixture of normal mouse and rabbit IgGs as a control, followed by washing in PBS-DS, and incubation for 1 h at room temperature with a cocktail of $F(ab')_2$ secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.): FITC-conjugated affinity-purified donkey anti–rabbit IgGs (5 μg/ml) (preadsorbed on bovine, chicken, goat, guinea pig, hamster, horse, human, mouse, rat, and sheep serum proteins); indodicarbocyanine (Cy5)-conjugated affinity-purified donkey anti–mouse IgGs (5 μg/ml) (preadsorbed on serum proteins from species above). After washing in PBS-DS, sections were mounted in slowfade medium (Molecular Probes), and viewed on a Zeiss Axiovert 35M microscope equipped with a laser scanning confocal attachment (MRC-1024; Bio-Rad Laboratories), using a 40× 1.3 NA objective lens. Fluorescent images were collected by using the 488 (for FITC) and 647 nm (for Cy5) excitation lines from an argon/krypton mixed gas laser. Color composite images were generated using Adobe Photoshop 4.0 by attributing the green and red color to either FITC- or Cy5-specific fluorophore spectra depending on experimental conditions. Images were printed with a Fujix Pictrography 3000 color printer. Each experimental condition was performed in triplicate. A number of 27 slides from human breast carcinoma and 11 slides for colon carcinoma were immunostained, with at least 20 microscopic fields scored per slide.

Determination of Protein Concentration

Protein concentration was determined by dye methods with a Bio-Rad Laboratories assay kit with BSA as the standard.

Reagents

mAbs against TIMP-2 (D52) (Shofuda et al., 1998), human MMP2 (2-22) (Kawano et al., 1997), human Ln-5 γ2 chain (D4B5) (Mizushima et al., 1998), and rat Ln-5 (MIG-1 and TR-1) (Plopper et al., 1996), and polyclonal antibody against rat Ln-5 γ2 chain (1963) were generated in our laboratories. Transwells were purchased from Corning-Costar; polyclonal antibody against human MT1-MMP (AB815) were purchased from Chemicon International, Inc.; protein A–Sepharose 4B was purchased from Pharmacia Biotech Sverige.

Results

We previously showed that, in breast epithelial cells, migration over Ln-5 is triggered by MMP2 (Giannelli et al., 1997). In that system, though, MMP2 must be activated and added exogenously, raising the question as to how general the mechanism might be, particularly in cell types that display constitutive migration over Ln-5. To address this question, we tested the effects of BB94, a hydroxamate compound known to broadly inhibit MMPs, in migration assays with a panel of epithelial cells that constitutively migrate on Ln-5 (Miyazaki et al., 1993b). In every case, migration on Ln-5–coated Transwell filters was inhibited by BB94 in a dose-dependent manner (Fig. 1). Similar levels of inhibition were achieved with TIMP-1 and TIMP-2, specific inhibitors of MMPs that are naturally occurring in tissues (Fig. 2 A). These data were a first indication that MMPs may be involved in the mechanism that elicits the constitutive migration of those cells on Ln-5.

In both migration (not shown) and scattering (Fig. 2 B) assays, the inhibitory activity of BB94 was abolished if Ln-5 had been cleaved with MMP2 before exposure to cells. Furthermore, the anti–Ln-5 antibody, MIG-1, inhibited scattering (Fig. 3 A) and blocked migration (Fig. 3 B) on Ln-5. These results further supported the possibility that constitutive migration on Ln-5 (i.e., without exogenous addition of activated MMPs) depends on cleaving Ln-5 with endogenously produced and activated MMPs.

To investigate this possibility further, we monitored MMP production by zymography in these cell lines. In Fig. 4, we show representative results with the cell line BRL, compared with HT1080 cells, known to secrete and activate pro-MMP2 (Shofuda et al., 1998; Stanton et al., 1998), and MCF-10A, a breast cell line that instead requires exogenously activated MMP2 to cleave and migrate on Ln-5 (Giannelli et al., 1997). Gelatin zymography of condi-

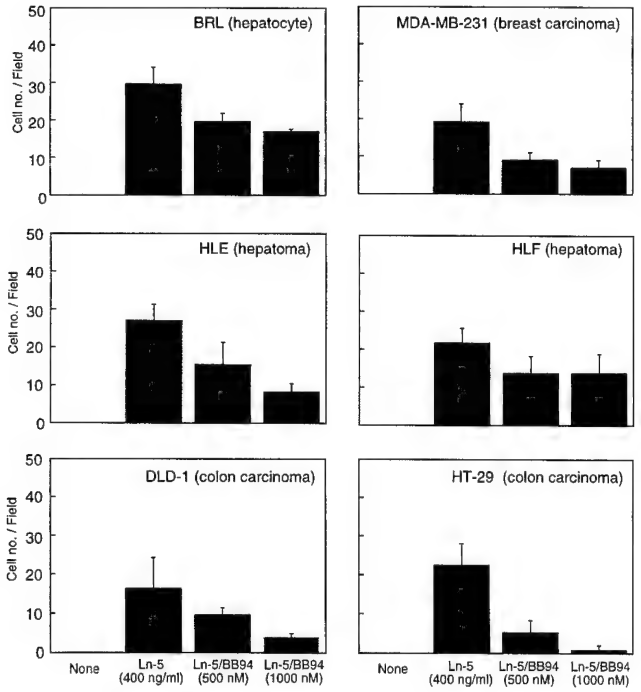

Figure 1. Effects of the hydroxamate MMP inhibitor BB94 on constitutive cell migration over Ln-5. Indicated cell lines were incubated under optimized conditions (see Materials and Methods for experimental details) in the upper compartment of Transwell chambers, which contained purified Ln-5 (200 or 400 ng/ml) in the lower compartment. Constitutive migration occurred in all cases. Likewise, BB94 blocked migration in a dose-dependent fashion. Each bar represents the mean + SD of three wells. The same results were obtained by coating the lower surface of Transwell filters with Ln-5, rather than adding soluble Ln-5 to the lower chamber.

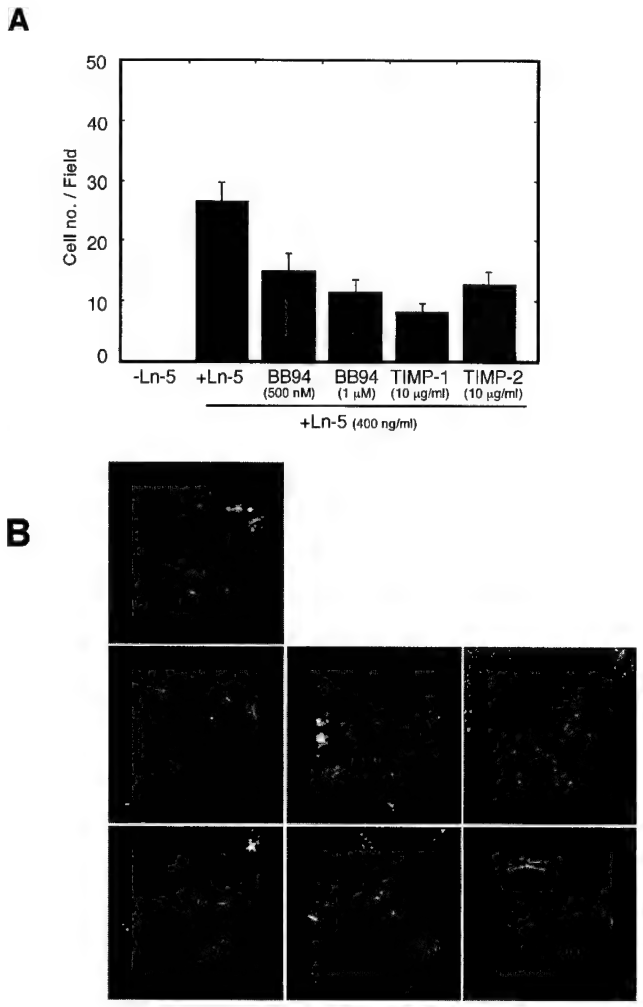

Figure 2. (A) Effect of TIMPs on BRL migration over Ln-5. BRL cells were incubated in Transwell chambers, in control medium or in the presence of Ln-5 (200 ng/ml) and the indicated concentrations of MMP inhibitors in the lower chamber. In the presence of Ln-5, BRL cells migrate constitutively. Both TIMP-1 and TIMP-2 inhibit migration, to levels comparable to BB94. Each bar represents the mean + SD for cell migration of two wells. (B) Lack of inhibition by BB94 of scattering induced by MMP2-cleaved Ln-5. BRL cells were incubated in 24-well culture plates in control medium (top), Ln-5 (80 ng/ml) (middle) or MMP2-cleaved Ln-5 (80 ng/ml) (bottom), in the presence of BB94, 500 nM or 2 μM. Cell morphology was examined under a phase-contrast microscope after incubation for 16 h. Scattering occurs with both intact Ln-5 and cleaved Ln-5. However, BB94 inhibits scattering induced by Ln-5, not by cleaved Ln-5, further supporting involvement of MMPs in the constitutive scattering of BRL cells.

tioned medium demonstrated that all three cell lines secrete a 72-kD gelatinolytic activity corresponding to pro-MMP2 (Fig. 4 A). However, the 62-kD–activated MMP2 could be detected only in HT1080, not in BRL or MCF-10A conditioned media (Fig. 4 A). Since MMP2 activation occurs at the cell surface, we next prepared plasma membranes from these cells and tested them by zymography. Two gelatinolytic bands at 72 and 62 kD, corresponding to the latent and activated form of MMP2, respectively, were detected in plasma membrane preparations from BRL and HT1080, but not MCF-10A cells (Fig. 4 B).

Cell surface activation of MMP2 may occur via a complex of pro-MMP2 with the tissue MMP inhibitor, TIMP-2, and the membrane-bound metalloprotease, MT1-MMP, as described previously (Strongin et al., 1995; Stanton et al., 1998). To confirm that BRL cells activate MMP2 via this mechanism, we tested for the presence of plasma membrane–associated TIMP-2 and MT1-MMP. By Western blotting, TIMP-2 was detectable in the conditioned media of all three cells. In contrast, only plasma membrane preparations from BRL and HT1080 cells, not MCF-10A cells (Fig. 4 C) contained TIMP-2. Similarly, membrane-associated MT1-MMP was clearly detectable in BRL and HT1080, and was very faint in MCF-10A cells (Fig. 4 D). (Note that, in addition, a 43-kD self-processed form of MT1-MMP [Stanton et al., 1998] was detected in the membrane of HT-1080 cells, [Fig. 4 D, asterisk].) These results suggested that constitutive migration on Ln-5 may depend upon secretion of MMP2 and expression of MT1-MMP and TIMP-2, which are required to activate MMP2.

Surprisingly, however, MMP profiling of the cell line panel from Fig. 1 showed that three of these cell lines do not secrete MMP2 (Table I), even though they constitutively migrate on Ln-5. Every migratory cell, however, was

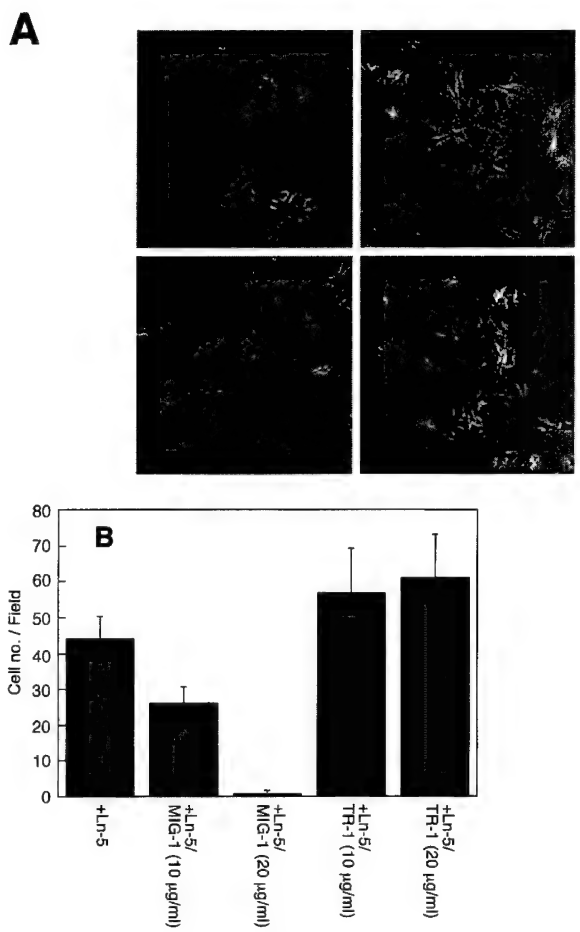

Figure 3. Inhibitory effects of mAb MIG-1 on Ln-5 BRL cell scattering and migration. (A) BRL cells were incubated in control medium (None) or Ln-5 (60 ng/ml) in the presence of mAbs to Ln5 TR-1 (control) or MIG-1 (20 μg/ml), which blocks migration induced by MMP2-cleaved Ln-5. Cell morphology was examined under a phase-contrast microscope after incubation for 16 h. MIG-1, not TR-1, inhibits scattering induced by Ln-5. (B) Transwell chamber migration of BRL cells, stimulated by Ln-5 (200 ng/ml), is blocked by MIG-1, not TR-1 control, in a dose-dependent fashion. Each bar represents the mean + SD of three wells.

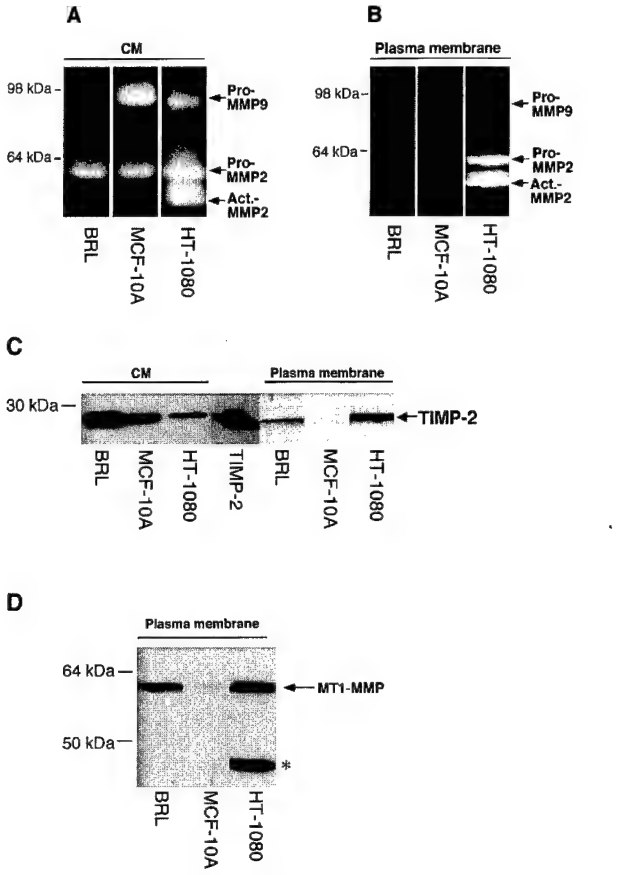

Figure 4. (A and B) Gelatin zymography of (A) conditioned media (CM) (60 μl/lane) or (B) crude plasma membrane fractions (2 μg/lane) from BRL, MCF-10A, and HT-1080 cells. The positions of pro-MMP9 (92 kD), pro-MMP2 (72 kD), and activated MMP2 (62 kD) are indicated. Note the presence of activated MMP2 in BRL, but not MCF-10A plasma membrane fractions. (C and D) Western blotting of BRL, MCF-10A, or HT-1080 conditioned media (CM) (300 μl/each lane) or crude plasma membrane fractions (2 μg/each lane) with antibodies to TIMP-2 (C) or MT1-MMP (D). In C, purified human TIMP-2 (50 ng) was run in the indicated lane. The respective positions of TIMP-2 and MT1-MMP are indicated to the right of gels. (asterisk) Processed form of MT1-MMP (see text).

positive for MT1-MMP, suggesting that this cell surface MMP may be directly involved in cleaving Ln-5 and promoting migration. Therefore, purified Ln-5 was incubated with recombinant, soluble MT1-MMP. This recombinant MT1-MMP preparation, produced in a bacterial expression system, was functional since it activated pro-MMP2 to the intermediate MMP2 form in vitro, as expected, and displayed proteolytic activity by gelatin zymography (data not shown). As shown in Fig. 5 A, recombinant MT1-MMP cleaved the γ2 subunit of Ln-5 in a dose-dependent manner. The cleavage products have the same molecular size of γ2′ (Marinkovich et al., 1992) and γ2x (Giannelli et al., 1997), respectively, two previously described proteolytically processed forms of the γ2 subunit (Fig. 5, B and C). No changes where observed in the size of the other two subunits of Ln-5, α3, and β3 (not shown). A detailed account of Ln-5 cleavage by MT1-MMP will be published elsewhere.

Taken together, these results suggested a model whereby constitutive migration on Ln-5 may be achieved in two manners: (1) by MMP2 secretion in conjunction with expression of MT1-MMP, which activates pro-MMP2 and leads to Ln-5 cleavage (Fig. 6 A); and (2) by expression of MT1-MMP alone, with no MMP2 secretion, since MT1-MMP can directly cleave Ln-5 and presumably cause mi-

Table I. Expression of MMPs and TIMP-2 in Plasma Membrane of Normal and Cancer Cells

	BRL	MCF-10A	MDA-MB-231	HLE	HLF	DLD-1	HT-29	HT-1080
MT1-MMP	++	±	++	++	+	+	+	+++
Pro-MMP2	++	−	−	+	+	−	−	+
Active MMP2	+	−	−	±	±	−	−	+++
TIMP-2	+	−	NT	+	+	NT	NT	++

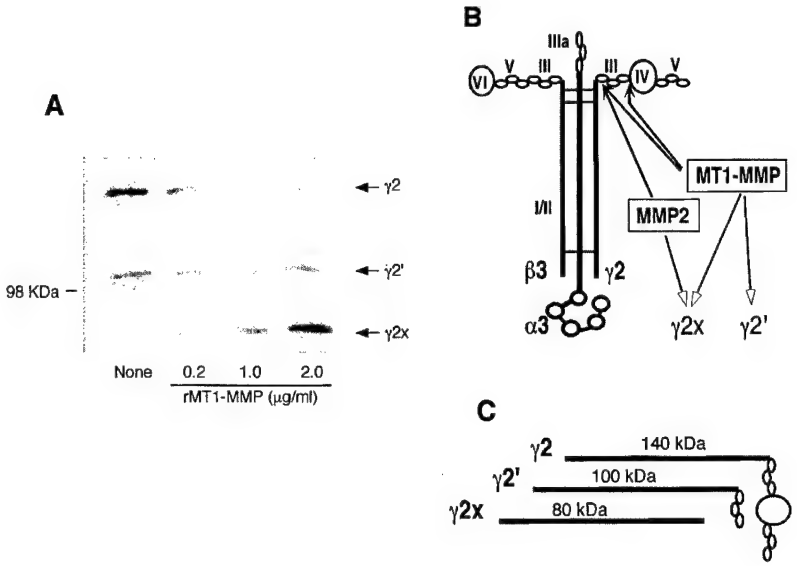

Figure 5. Cleavage of Ln-5 by MT1-MMP. (A) Ln-5 (1 μg) was treated with recombinant soluble MT1-MMP at the indicated concentrations, electrophoresed by 6% SDS-PAGE under reducing conditions, and then analyzed by Western blotting with a polyclonal antibody (1963) against rat Ln-5 γ2 chain. The positions of Ln-5 γ2 (140 kD), γ2′ (100 kD), and γ2x (80 kD) chains are indicated. In the presence of increasing concentrations of MT1-MMP, the γ2 appears to chase into the γ2′ and the γ2x chain. (B) Schematic depiction of the position of the MMP2 cleavage site (Giannelli et al., 1997) and the predicted position of the MT1-MMP sites. The γ2′ has been described (Vailly et al., 1994). (C) Scheme of the predicted size of MMP2 and MT1-MMP cleavage products of the Ln-5 γ2 chain.

gration (Fig. 6 B). To test this model, we diminished the expression of MT1-MMP in BRL and HT-29 cells by treatment with rat or human MT1-MMP antisense oligonucleotides, respectively.

In cells treated with MT1-MMP antisense, but not with control, scrambled oligonucleotides, expression of surface MT1-MMP was reduced by ~67% in HT-29 cells and 40% in BRL cells (compared with β-actin internal controls), as determined by Western blotting followed by scanning densitometry on a Molecular Dynamics FluorImager (not shown). Importantly, in the antisense-treated cells, there was >50% inhibition of constitutive Ln-5 migration (Fig. 7 A), as well as scattering (not shown).

As expected, inhibition was observed both in the MT1-MMP+, MMP2+, and in the MT1-MMP+, MMP2− cell lines. Furthermore, addition of MMP2-cleaved, rather than intact Ln-5, to antisense-treated cells circumvented the inhibition of constitutive Ln-5 migration (not shown) or scattering (Fig. 7 B), supporting a direct relationship between MT1-MMP expression and Ln-5 cleavage.

In a further test, we used the MMP2 hemopexin-like domain (HLD), which inhibits activation of pro-MMP2 by competitively binding to TIMP-2 and preventing formation of the activating complex with MT1-MMP (Strongin et al., 1995). However, HLD is not known to interfere with MT1-MMP enzymatic activity. HLD significantly inhibited Ln-5 migration of BRL cells (Fig. 8). In contrast, no inhibition was observed for HT-29 cell migration on Ln-5 (Fig. 8). These results are in agreement with our working model (Fig. 6) that constitutive Ln-5 migration depends on both MT1-MMP and MMP2 in BRL cells, whereas, in HT-29, cells are dependent on MT1-MMP only.

To evaluate the possible relevance of these mechanisms in vivo, we immunostained with antibodies to Ln-5, MT1-MMP, and MMP2 sections of human breast (not shown) or colon cancer tissues (Fig. 9). Double immunofluorescence and confocal microscopy indicated that Ln-5, which was expressed predominantly at the outer edge of cancer cell nests, colocalized with either MT1-MMP or MMP2 at many locations (Fig. 9, arrows). These results are consistent with the possibility that Ln-5 cleavage, and consequent induction of migration, occurs at defined locations corresponding to areas of the cell surface where either MT1-MMP or activated MMP2 are expressed.

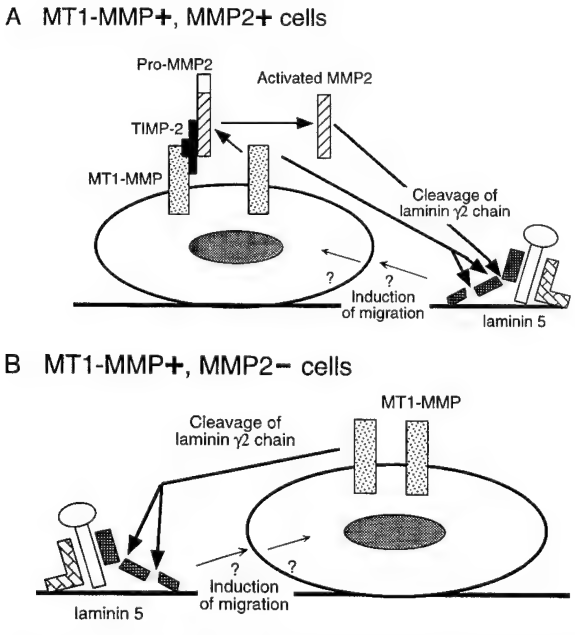

Figure 6. Schematic model for mechanisms of MMP-dependent Ln-5 cell migration.

Discussion

We report that Ln-5 cleavage by MMPs may be a widespread mechanism that triggers cell migration. This conclusion is based on the following findings: (1) in several cell lines that migrate (or scatter) constitutively on Ln-5, migration (or scattering) on Ln-5 was blocked by inhibitors of MMPs, both naturally occurring, like TIMPs, or synthetic, like BB94; (2) in these cell lines, the ability to

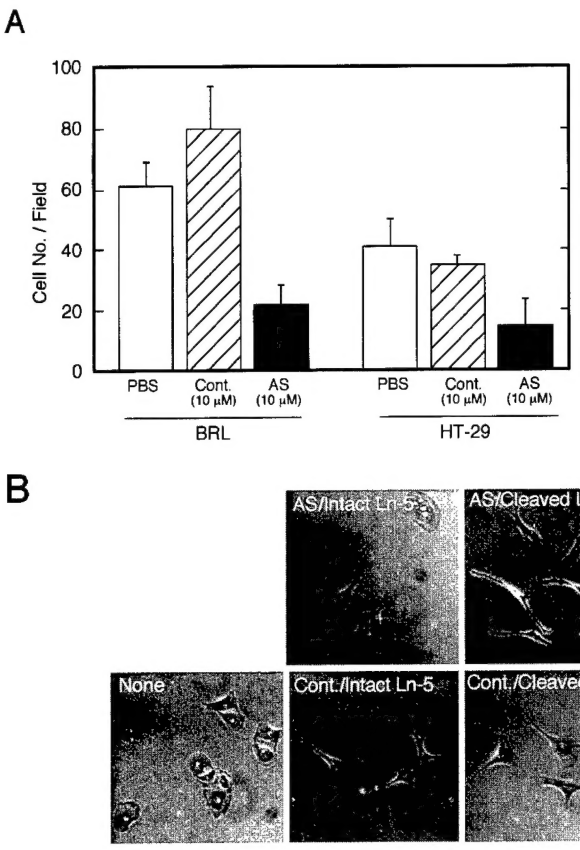

Figure 7. Effect of MT1-MMP antisense oligonucleotides on Ln-5 migration by BRL or HT-29 cells. (A) Cells were pretreated for 2 d in culture with MT1-MMP antisense (AS) or control, scrambled (Cont.) oligonucleotides, and tested in migration assays. Ln-5 and oligonucleotides, at the same concentrations as treatment, or PBS were present throughout the assay in the lower Transwell chambers. Each bar represents the mean + SD for cell migration of two (BRL) or four (HT-29) wells. (B) BRL cells were pretreated with MT1-MMP AS or control oligonucleotides, and tested in the scattering assay as in Fig. 2, in the presence of either intact or MMP2-cleaved Ln-5. Addition of either intact or MMP2-cleaved Ln-5 causes a morphological change in BRL cells, which loosen cell–cell contacts, elongate, and scatter. MT1-MMP AS treatment inhibits these morphological changes when they are induced by intact Ln-5. In contrast, cleaved Ln-5 still causes scattering of AS-treated cells, supporting a link between reduction of MT1-MMP expression, Ln-5 cleavage and scattering response.

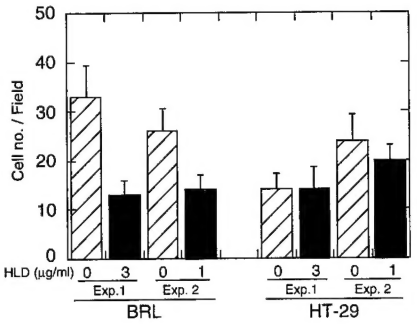

Figure 8. Effect of MMP2 HLD on Ln-5 migration by BRL or HT-29 cell. Purified HLD was added to the lower Transwell chambers at 0, 1, or 3 μg/ml. Results shown are from two separate, representative experiments (1 and 2) performed on different occasions. Each bar represents the mean + SD for cell migration of four wells.

migrate constitutively on Ln-5 correlated with expression of membrane bound MT1-MMP; (3) decreasing expression of MT1-MMP via antisense oligonucleotides inhibited migration; and (4) purified Ln-5 itself was cleaved in vitro by MT1-MMP with a pattern similar to MMP2, thus, providing a mechanism for induction of migration (Giannelli et al., 1997).

These findings significantly extend the physiological implications of our previous report that cleavage of Ln-5 by MMP2 may induce migration (Giannelli et al., 1997). In that report, we had shown that adding exogenously activated MMP2 to purified Ln-5 changed the latter into a substrate that triggered cell migration. The only indication that the mechanism may operate in vivo was that Ln-5 fragments, similar to those generated by MMP2, were detectable in remodeling, but not in quiescent tissues. Here we show that, in fact, cells that have the constitutive ability to migrate on Ln-5 do so by using an MMP-dependent mechanism. MMP2 was secreted and activated by several of the constitutive migratory cells. This MMP also contributed to stimulating migration, since its HLD fragment, which blocks its activation, inhibited migration. However, inhibition was only partial in MT1-MMP+, MMP2+ cells, and nondetectable in MT1-MMP+, MMP2− cells. Together, these results suggest a model whereby one preferred mode for stimulating migration on Ln-5 is via MMP cleavage of Ln-5 itself. Cleavage may be carried out by MT1-MMP alone or in concert with MMP2. Since MT1-MMP is required to activate MMP2, it is likely that MT1-MMP plays an essential role in this mechanism, whereas MMP2 may represent a potentiation loop. An attractive feature of this model is that, whether Ln-5 is cleaved by MT1-MMP alone or by MT1-MMP–activated MMP2, the proteolytic components are anchored onto the cell surface because MT1-MMP is a transmembrane protein. Thus, spatially directed cleavage of Ln-5 may occur, perhaps coincident with hot spots for migration.

By incubating recombinant MT1-MMP with purified Ln-5, we showed, for the first time, that Ln-5 is a proteolytic substrate for MT1-MMP (Fig. 5). While the details of this cleavage are being currently characterized and will be published elsewhere, it is clear that only the γ2 subunit of Ln-5 is cleaved by MT1-MMP, whereas the α3 and β3 subunits remain intact. The Ln-5 γ2 subunit is synthesized as a 135-kD polypeptide which is proteolytically processed (Vailly et al., 1994) by removal of 434 NH_2-terminal amino acids (γ2′). The enzyme that carries out this processing is not known, but our preliminary results indicated that it may be MT1-MMP. This would be consistent with the pattern generated by digestion of purified Ln-5, showing a time-dependent increase in γ2′ (Fig. 5). MMP2 can cleave both γ2 and γ2′, yielding an 80-kD γ2x chain (Giannelli et al., 1997). It is clear that MT1-MMP can also produce the γ2x fragment (Fig. 5). Thus, we predict two MT1-MMP cleavage sites on Ln-5 (Fig. 5, B and C).

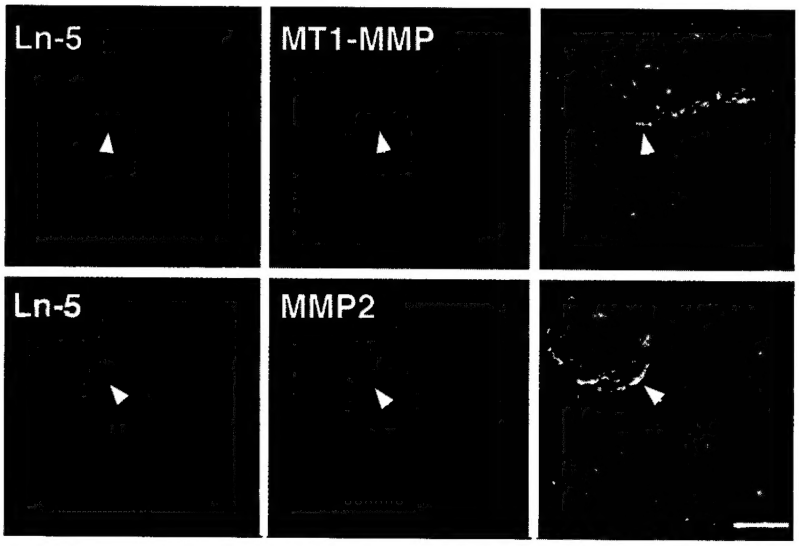

Figure 9. Detection of Ln-5, MT1-MMP, and MMP2 in human colon carcinoma tissue specimens by double immunofluorescence and confocal microscopy. Cryostat sections were double immunostained for either Ln-5 and MT1-MMP or Ln-5 and MMP2. Expression of Ln-5 is discrete, in some cases limited to the outside layer of cell nests. Colocalization with MT1-MMP and MMP2 is shown in the panels on the right (yellow) by combining the specific fluorophore spectra recorded for Ln-5 (green) and MT1-MMP or MMP2 (red). Examples of colocalization are indicated by arrowheads. A slight difference in the appearance of Ln-5–specific immunoreactivity in top and middle panels is due to the use of either mouse mAb D4B5 (combination with rabbit anti–MT1-MMP) or rabbit polyclonal 1963 (combination with mouse monoclonal to MMP2). Control sections incubated with normal mouse IgGs and rabbit IgGs were completely negative (not shown). Bar size, 43 μm.

Miyazaki and colleagues described ladsin, a soluble protein in the spent media of several epithelial cell lines, that induced scattering and migration within hours upon addition to cultured cells (Miyazaki et al., 1993b). Ladsin turned out to be identical to Ln-5 (Mizushima et al., 1996), raising the following apparent conflict: in epithelial tissues and in certain culture systems, Ln-5 promotes static adhesion of epithelial cells via formation of hemidesmosomes; in contrast, ladsin promotes an opposite effect, scattering. Our results provide an explanation for this apparent conflict and reconcile the data in the literature. Thus, MT1-MMP+ cell lines are capable of cleaving Ln-5 into its migratory form, directly and via activation of MMP2, if present. In contrast, MT1-MMP− cells leave Ln-5 intact and use it for static adhesion.

To date, MT1-MMP was only known to digest collagens I, II, and III. Our results add new perspective in at least two respects. First, the substrate Ln-5 is located in the BM, in direct contact with epithelial cells, which are anchored to it via receptor integrins (α3β1 or α6β4). In contrast, epithelial cells do not express receptors for collagen IV (integrin α1β1) and, as far as we are aware, there is no report of MT1-MMP cleaving collagen IV or of promigratory activity by collagen fragments. Thus, it is possible that, in the context of the BM, the Ln-5 substrate may play a prominent role in mediating MT1-MMP effects on epithelial cells. Second, because the Ln-5 cleavage mechanism may work in tissue remodeling and repair (Giannelli et al., 1997, 1999), it is expected to be spatially constrained. This requirement may be fulfilled by MT1-MMP, which is transmembrane-anchored and presumably does not diffuse freely in the extracellular space, so that it may be targeted at discrete BM sites by cell surface contact. In addition, MT1-MMP may also constrain spatially MMP2, which is secreted extracellularly, by recruitment via TIMP-2 (see below).

All evidence indicates that upon addition to constitutive migratory cell types, Ln-5 is cleaved by MMPs. However, in spite of extensive efforts, we were not able to detect cleaved Ln-5 in these cultures. This is not entirely surprising, in view of the fact that relatively small amounts of Ln-5 molecules may gain access to relevant cell surface sites, and nonetheless exert their biological effect when MMP cleaved. More sensitive detection methods may shed light on this point. Previously, it was reported that cleavage of the Ln-5 α3 chain by plasmin correlated with nucleation of hemidesmosomes (Goldfinger et al., 1998), presumably inhibiting cell migration. Therefore, it is possible that plasmin and metalloproteases act coordinately to regulate epithelial cell migration on Ln-5.

MMPs are involved in tissue remodeling under various physiological and pathological conditions such as morphogenesis, angiogenesis, inflammation, tissue repair, and tumor invasion (Matrisian, 1992; Stetler-Stevenson et al., 1993). In particular, inflammatory macrophage and malignant cancer cells secrete MMP2 to degrade BM and connective tissue ECM to invade. Deryugina et al. (1997) have shown that MMP2 activation and integrin αvβ3 modulate glioma cell migration on ECM. MMP2 also modulates melanoma cell attachment and facilitates migration and invasion (Ray and Stetler-Stevenson, 1995). Ln-5 also has been associated with carcinoma cell invasion. In particular, the γ2 chain of Ln-5 was detected at the leading edge of invasive colon and breast cancer tissue (Pyke et al., 1995; Sordat et al., 1998). In our study, migration of hepatoma, breast and colon carcinoma cells was induced by Ln-5, via the MMP cleavage mechanism. Furthermore, colocalization of Ln-5 with MT1-MMP and MMP2 was detected in breast and colon cancer tissue. Thus, our results suggest a mechanistic framework for the observed association between MMPs and invasion, or Ln-5 and invasion. We propose that MT1-MMP cleavage of Ln-5 is a candidate to play a role in the early phases of tissue invasion, e.g., when carcinoma in situ may still be dependent on external factors to initiate local invasion. These issues are deserving of further investigation because of their obvious relevance to cancer progression.

Our results raise several questions concerning the regu-

lation of motility of epithelial cells in contact with Ln-5. In particular, it is important to determine how MMPs may be deployed. An attractive possibility is that epithelial cells may be stimulated to synthesize MT1-MMP by environmental signals and factors in situations that require remodeling, e.g., steroid-induced branching morphogenesis (Yu et al., 1997; Kadono et al., 1998). Alternatively, MMPs may be delivered to remodeling sites by third party cells, stromal or inflammatory. These mechanisms may also be exploited, inappropriately, by invading carcinoma cells. Another important issue concerns the nature of the cellular interactions with cleaved Ln-5, which trigger migration. Integrins are likely to play an important role because they can mediate adhesion and migration. Additional studies are now necessary to further our understanding of these molecular mechanisms of invasion, both in normal and neoplastic tissues.

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